Nucleic Acids and Protein Content as a Measure to Evaluate the Nutritional Condition of Japanese Flounder *Paralichthys olivaceus* Larvae and Juveniles

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Abstract

Recently developed fluorescence techniques were used to quantify RNA and DNA content in the whole body of fed and starved hatchery-reared larval and juvenile Japanese flounder. RNA/DNA ratios in wild larvae and juveniles were concurrently measured to evaluate their nutritional condition. Significant differences in the RNA/DNA ratios were found between fed and starved fish, and appeared to expand drastically as starvation proceeded. Even in the fed fish marked fluctuations in its ratios during metamorphosis were observed, evident by decreasing from late metamorphic to post-metamorphic stages. The changes in protein content coincided well with RNA content. Protein/DNA ratio also peaked at the post-metamorphic phase and decreased for several days thereafter, suggesting an occurrence of hypertrophy until the post-metamorphic phase followed by hyperplasia. Using the criteria established from these laboratory experiments, the nutritional conditions of wild Japanese flounder larvae and juveniles collected in Wakasa Bay in 1994 and 1995 were determined by measuring RNA and DNA. Starved fish were mainly found at stage I (settling stage) fish during the late season of settlement in 1995. The present study demonstrates the usefulness of RNA/DNA ratios and protein content for assessment of the nutritional condition of hatchery-reared and wild Japanese flounder larvae and juveniles.

Japanese flounder *Paralichthys olivaceus* is a commercially important species for mariculture and stock enhancement as well as coastal fisheries in Japan. Although a large variety of work has been done on the early life stages of Japanese flounder (Minami, 1982; Seikai *et al.*, 1986; Fukuhara, 1986), less is known about the mechanisms of its mortality in the sea (Tanaka *et al.*, 1989a). Hjort (1914) first proposed the critical period hypothesis, whereby the year class size in most marine fish species hatched from small pelagic eggs could be determined primarily from high mortality rates during early life stages. Consequently, small changes of survival rate during this time can give rise to high variability in recruitment (Sale, 1990; Fogarty, 1993). Over the years, laboratory studies have mainly focused on mortality during the first feeding stage of marine fish larvae (May 1974). More recently, however, several papers have begun to address the metamorphic phase of fish development as a critical period for survival (Thorisson, 1994). Recent works in flatfish particularly have tried to elucidate the possibility of a second severe mortality event during the post-metamorphic phase of settlement (Tanaka *et al.*, 1989a; Phil, 1990; Keefe and Able, 1993). Thus, the early larval stage would be a crucial period for survival and potential occurrence of another species-specific critical period can be predicted in association with metamorphosis in the Japanese flounder.

A variety of techniques such as morphometric, histological, and biochemical analyses for diagnosing the nutritional condition of fish larvae and juveniles have been developed and applied to both hatchery-reared and wild fish (Buckley, 1979; Yin and Blaxter, 1986; Clemmesen, 1987; 1988; Theilacker and Watanabe, 1989). Biochemical analysis, which determines the quantities of chemical constituents that serve as energy substrates, could be one of the indicative measures to show changes of nutritional conditions. Among the biochemical indices, the ratio of RNA to DNA has been proven a reliable indicator of nutritional condition (Buckley, 1979; 1980) and growth of larval and juvenile fish (Bulow, 1970). Tanangonan *et al.* (1998) did preliminary studies of biochemical changes for hatchery-reared Japanese flounder. However, more detailed experiments are needed to assess nutritional condition of Japanese flounder associated with development and apply the biochemical criterion to the wild fish.

In the present study the RNA, DNA, and protein content of wild and hatchery-reared larval and juvenile Japanese flounder are investigated to evaluate nutritional condition and the usefulness of such techniques.

Materials and Methods

Japanese flounder larvae and juveniles were reared at the Fisheries Research Station of Kyoto University, Maizuru, Japan, in April and May 1994. Fertilized eggs, provided by the Japan Sea Farming Association (Miyazu Station), were stocked into 500-L polycarbonate tanks, and maintained at 15 C using running seawater. Within six hours of stocking, water temperature was raised to an average of 18 C using heating rods connected to a thermostat. Larval rearing was conducted under natural photoperiod conditions. The larvae were fed rotifers (*Brachionus plicatilis*) cultured with *Nannochloropsis oculata* at three days after hatching (DAH), and brine shrimp (*Artemia salina nauplii*) enriched with squid liver oil at 20 DAH. Rotifers were provided at a density of five ml^{-1} until 28 DAH, and brine shrimp were given until 43 DAH at a density of one to six ml^{-1} every morning.

In Experiment (Exp.) 1 RNA/DNA ratios showed large fluctuations at stages G, H, and I due to failure in determining the sample amount from the homogenized one. In order to confirm the values of RNA/DNA ratios at specific stages during metamorphosis, a supplemental, second experiment was performed under almost identical rearing conditions as the first. Experiment 2 lasted for 15 days between the early-metamorphosing stage (F) and eight days after settlement (I_8).

To reconfirm the changes in RNA/DNA ratios during metamorphosis and settlement, a rearing experiment lasting for 21 days from E to I_8 stage (eight days after reaching I stage) was designed and carried out under similar rearing conditions to the previous experiments. To initially reduce individual variance, approximately 3,000 larvae at E stage were carefully selected from the 500-L stocking tanks and transferred into 100-L rearing tanks. Development of Japanese flounder larvae and juveniles was classified into nine developmental stages according to Minami (1982), and its metamorphic process was grouped into three phases (Gwak *et al.*, 1999).

The starvation phase involved 300 fish at each developmental stage from A to E carefully sorted from the three 500-L stocking tanks during Exp.1 and transferred into 100-L tanks. Three hundred fish from the F stage were also selected during the second experiment and transferred into 100-L tanks. The fish were kept in circulated seawater with aeration at 18 C without food.

The starvation experiment ended on the day of 100% mortality based on the result of a previous point-of-no-return study by Gwak *et al.* (1999).

Samplings for fed and starved fish at each developmental stage were done during 43 days from 3 DAH for the fed group and just before the day of 100% mortality for the starved group (Table 1). To determine quantities of DNA and RNA in the entire body, six fish were individually sampled, rinsed, pipetted into Eppendorf micro vials, immediately frozen at -25 C, and stored at -87 C until analysis. All the samplings and preservations for RNA/DNA ratios were made from both fed and starving groups during the rearing experiment. In Exp. 2 the sampling and preservation was done in the same manner.

Table 1. Criterion established by RNA/DNA ratio for determining the nutritional conditions of Japanese flounder larvae and juveniles.

Stage	Body Length (mm)	Characters	Metamorphic Phases	Metamorphosing Stages	Nutritional Status Dying ≤ starving* ≤ healthy
A	3.6	First feeding	Premetamorphic		1.48 – 2.15
B	4.4				1.34 – 2.15
C	5.7				2.13 – 3.19
D	7.4				1.16 – 3.50
E	7.7	Metamorphosis	Metamorphic	Early-	1.56 – 4.03
F	8.3				1.20 – 2.52
G	9.3			Mid-	1.08 – 3.24
H	11.4			Late-	1.69 – 5.36
I	11.6	Settlement	Postmetamorphic		1.35 – 3.78

** The range of RNA/DNA ratio that can be classified as a starving.*

To determine the quantities of RNA, DNA, and protein content in the whole body during the third experiment, ten additional fish were sampled and stored until later analysis. For measurements of dry weight individual fish were placed on pre-weighed pieces of foil and dried to a constant weight at 60 C. To obtain a precise value of dry weight, especially for the smallest larvae, freshly activated silica gel was placed in the balance to control for relative hygrometry. A minimal amount of time spent from removal from the desiccator to weighing was controlled strictly.

Field collections were performed in Wakasa Bay, Sea of Japan using a net 1.3 m in diameter and 0.33 mm mesh for the pelagic larvae and a beam trawl (2.0 m mouth width and 4.0 mm mesh) for the settled juveniles during the settling season from March to June in 1994 and 1995. Temperature and salinity were measured at each site with a Yellow Springs International (YSI) SCI Meter 33. A total of 187 larvae and juveniles (Table 2) were analyzed for their individual RNA/DNA ratios. Since tissues of fish larvae deteriorate quickly due to autolysis (Theilacker 1978), individual larvae and juveniles were stored on dry ice immediately after collection. To determine if these animals were in starving condition, RNA/DNA ratios were compared to the values determined from the larvae and juveniles starved under laboratory conditions shown in Table 1. The samples with RNA/DNA ratios below these values were considered "starving."

Table 2. Percentage of starving larvae and juveniles in each sampling date for 1994 and 1995.

Sampling Date	Total Catch	Stage	No.	No. of Starving	% Starving	Depth (m)	Temp (ᵊC)
13 May 1994	36	C	11	2	18	20-40	17
		D	10	2	20		
		E	13	0	0		
		G	2	0	0		
20 May	39	B	4	0	0	20	17
		C	16	0	0		
		D	7	0	0		
		E	7	0	0		
		F	4	0	0		
		G	1	0	0		
6 April 1995	1	F	1	0	0	113	15
21 April	8	G	4	0	0	10	16
		H	2	1	50		
		I	2	1	50		
8 May	54	F	2	0	0	3.5-10	17
		G	18	0	0		
		H	19	0	0		
		I*	15	1	0		
25 May	34	I	34	22	65	4.5-5	17
5 June	15	F	2	0	0	3.5	18
		G	2	0	0		
		I	11	10	91		

** I stage fish that are newly or recently settled.*

The quantity of RNA and DNA in the whole body from A to E stage was determined individually by using a specific nucleic acid fluorescent dye, Ethidium Bromide, developed by Clemmesen (1993), and slightly modified by Sato *et al.* (1995). In F stage, nucleic acids were extracted from the whole body by homogenizing the larvae and juveniles in Tris-HCl buffer (0.05 M Tris, 0.1 M NaCl, 0.01 M EDTA, pH 8.0), using a glass homogenizer placed in 4 C ice-cold water. In order to measure the DNA content of a sample, RNA was enzymatically digested with RNase and the remaining DNA was determined with Ethidium Bromide. Salmon sperm DNA (Wako Pure Chem. Co., Ltd.) and yeast RNA (Kanto Chem. Co., Ltd.) were used as standards.

A Bio-Rad protein kit, using bovine serum albumin as a standard determined total protein dissolved in NaOH. Expressed as µg of protein per fish, the ratio of RNA to protein and protein to DNA are cited as indices of protein synthesis capacity and cell size, respectively (Mathers *et al.*, 1994). A one-way ANOVA (Fisher) was used for statistical evaluation. Significance was accepted for $P<0.05$.

Results

Developmental Changes

Figure 1 shows the developmental changes in RNA and DNA contents obtained from the Exp. 1. RNA and DNA contents of fed larvae increased with age, although the values largely fluctuated at G, H, and I stages. In Exp. 2, DNA contents increased gradually with age and showed a small peak just after settlement (on 34 DAH). The RNA content showed a drastic increase between the mid and late metamorphic stages (G to H), but almost no increment between early and mid metamorphic stages (F to G). The RNA content decreased between post-metamorphic phase (I stage) and 39 DAH, but repeatedly increased thereafter (Fig. 2).

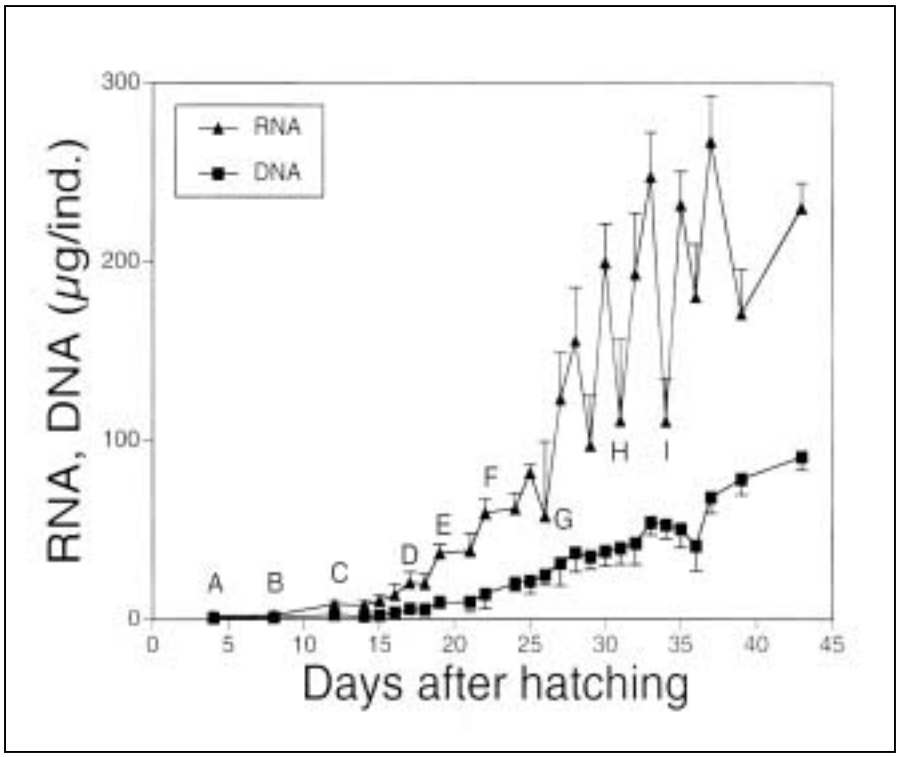


Figure 1. Developmental changes in RNA and DNA contents in fed larvae and juveniles of laboratory-reared Japanese flounder. Data points are mean values of six fish. A to I indicate the developmental stage.

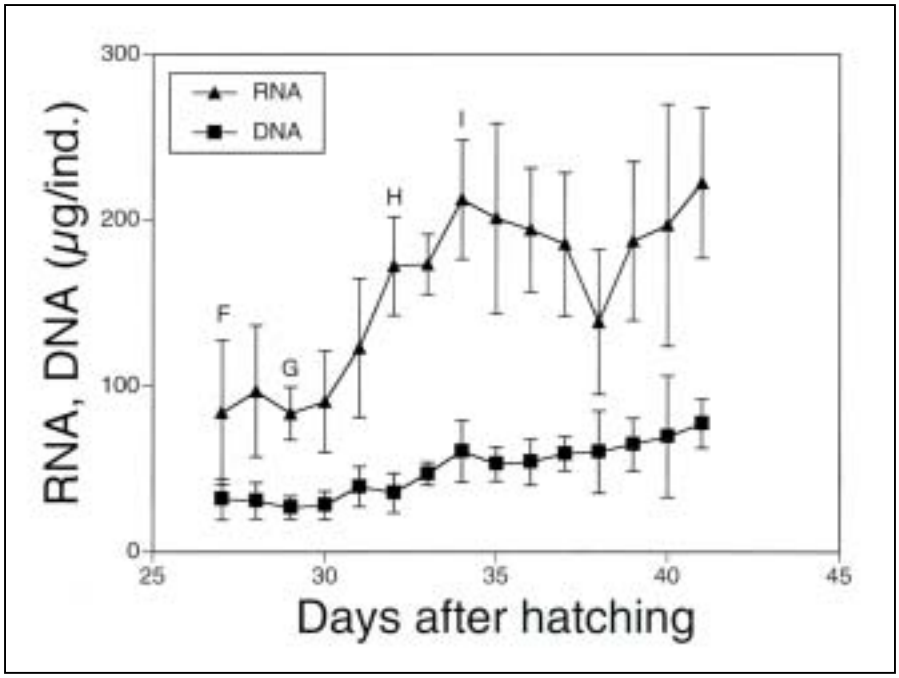


Figure 2. Ontogenetic changes in RNA and DNA contents of laboratory-reared Japanese flounder larvae and juveniles during metamorphosis (supplemental 2nd experiment). Data points are mean values of six fish. Vertical bars denote standard deviation, and F to I represent the developmental stage.

In Exp. 3, dry weight of larvae and early juveniles increased exponentially with development, enlarging individual variance in I stage (Fig. 3). These results indicate that changes in RNA and DNA content during metamorphosis were similar to those of Exp.2 (Fig. 4a). Ontogenetic changes in DNA and RNA content in terms of µg/mg DW (Fig. 4b) showed a very different pattern from those individually. The DNA content (µg/mg DW) at the early and mid stage appeared rather stable compared to the RNA content having an evident peak. Then both DNA and RNA contents showed a marked decrease until the post-metamorphic phase (I stage) before stabilizing again.

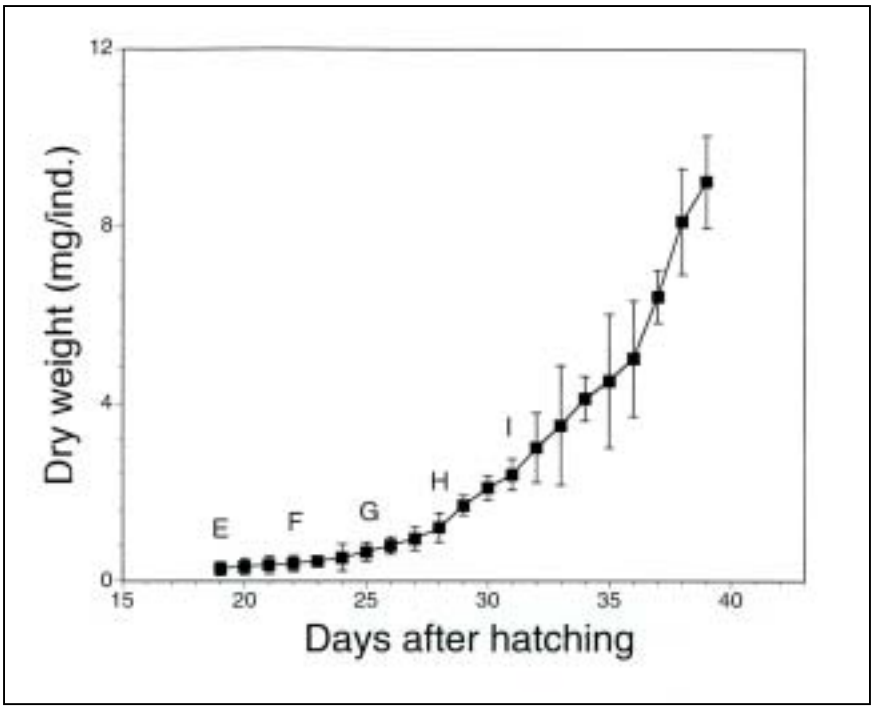
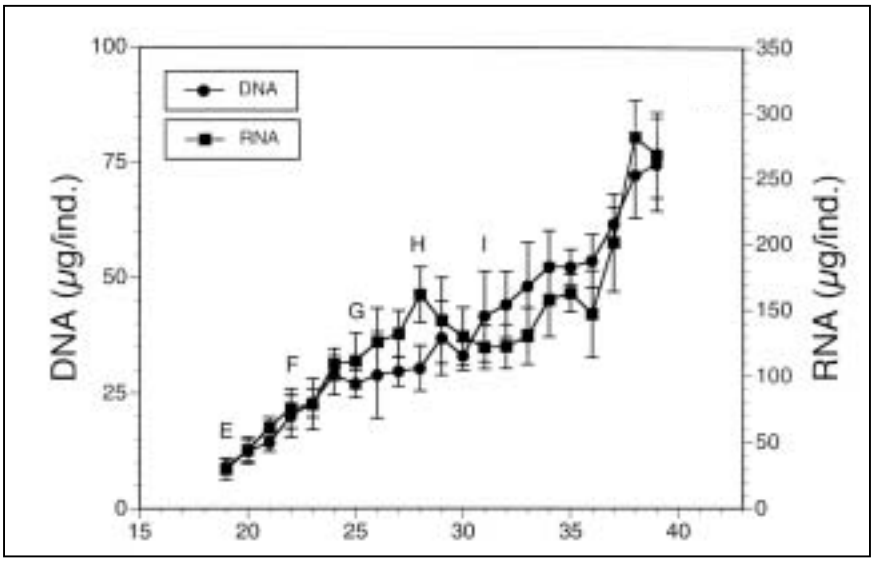


Figure 3. Developmental changes in dry weight (mg) of Japanese flounder larvae and juveniles during metamorphosis and early post-metamorphic phase (supplemental 3rd experiment). Values are given as the mean ± SD of twenty-five to thirty fish. E to I indicate developmental stage.

Figure 4a. Developmental changes in DNA and RNA contents of Japanese flounder larvae and juveniles during metamorphosis and early postmetamorphic phase (supplemental 3rd experiment) in :g/ind. (A) and :g/mg DW (B). Values are given as the mean ± SD of five to eight fish. E to I indicate developmental stage.



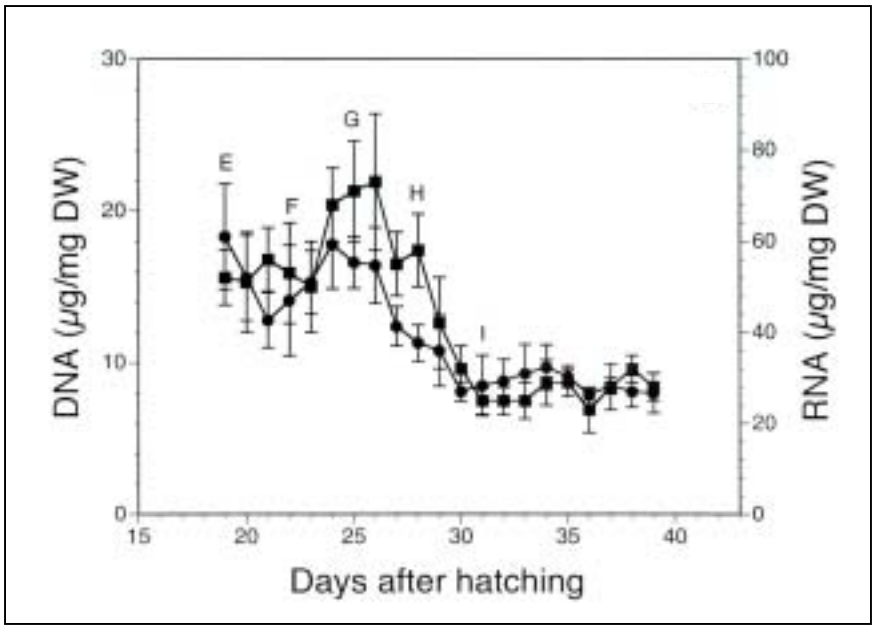


Figure 4b. Developmental changes in DNA and RNA contents of Japanese flounder larvae and juveniles during metamorphosis and early post-metamorphic phase (supplemental 3rd experiment) in :g/ind. (A) and :g/mg DW (B). Values are given as the mean ± SD of five to eight fish. E to I indicate developmental stage.

The protein content, as µg/ind., showed an overall increase between the early and late metamorphic stages, then stabilizing until reaching the I_2 stage (A in Fig. 5). After the post-metamorphic phase, the protein content of newly settled juveniles showed a drastic increase. A significant increase in protein content, as µg/mg DW, between the early and mid metamorphic stages was followed by a consistent decrease until the post-metamorphic phase (B in Fig. 5). Thereafter the protein content showed a gradual increase.

Figure 6 illustrates the relative amounts of DNA of the fed and starved larvae, with variability during development: lower in the fed larvae at D, E and H stages; higher at F, G, and I stages; and similar at A, B and C stages. The RNA content of the fed larvae exponentially increased with development, particularly accelerating at later phases of metamorphosis (G and H stages, Fig. 7). In contrast, the RNA contents in the starved larvae showed drastic reduction throughout the experiment with the onset of food deprivation. The rate of daily reduction appeared to be higher during the earlier days of starvation. Difference in RNA contents between fed and starved groups significantly increased as starvation proceeded, contrasting with DNA content that increased under starvation at several developmental stages.

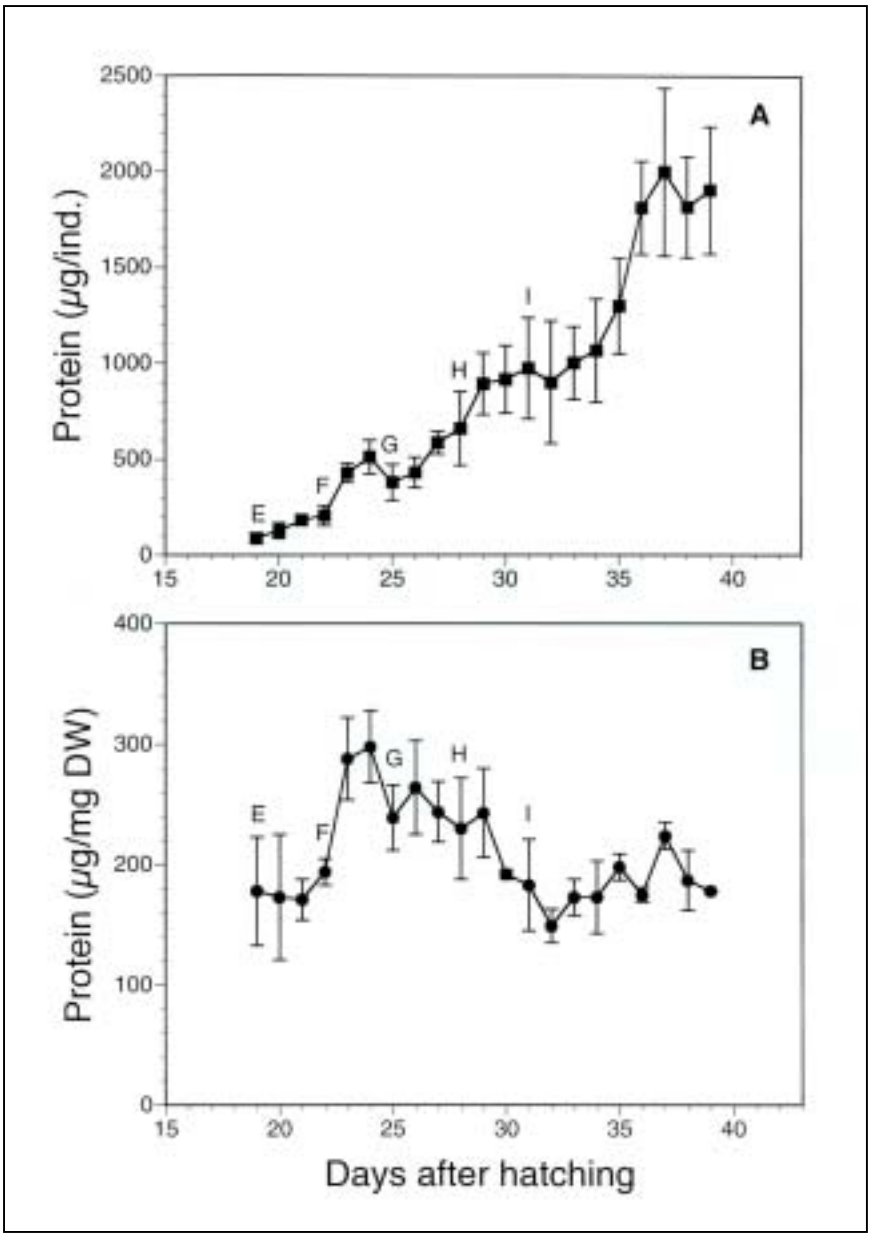


Figure 5. Developmental changes in protein content of Japanese flounder larvae and juveniles during metamorphosis and early post-metamorphic phase (supplemental 3rd experiment) in :g/ind. (A) and :g/mg DW (B). Values are given as the mean ± SD of five to eight fish. E to I indicate developmental stage.

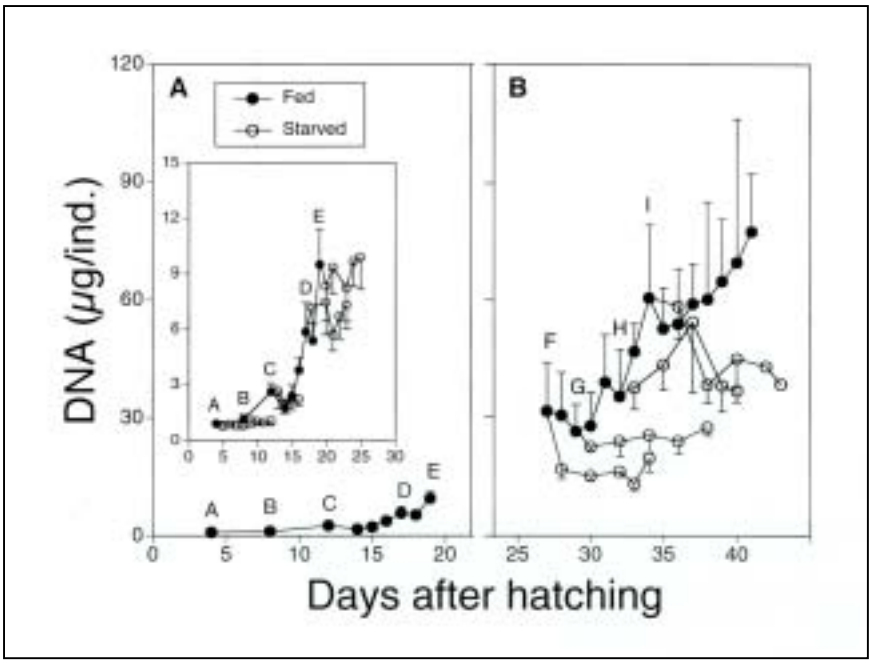


Figure 6. Developmental changes in DNA content of fed larvae and juveniles, and effects of starvation on the content in 1st (A) and 2nd (B) laboratory-reared Japanese flounder. Each point is the mean value of six fish. Vertical bars denote standard deviation, and A to I represent the developmental stage.

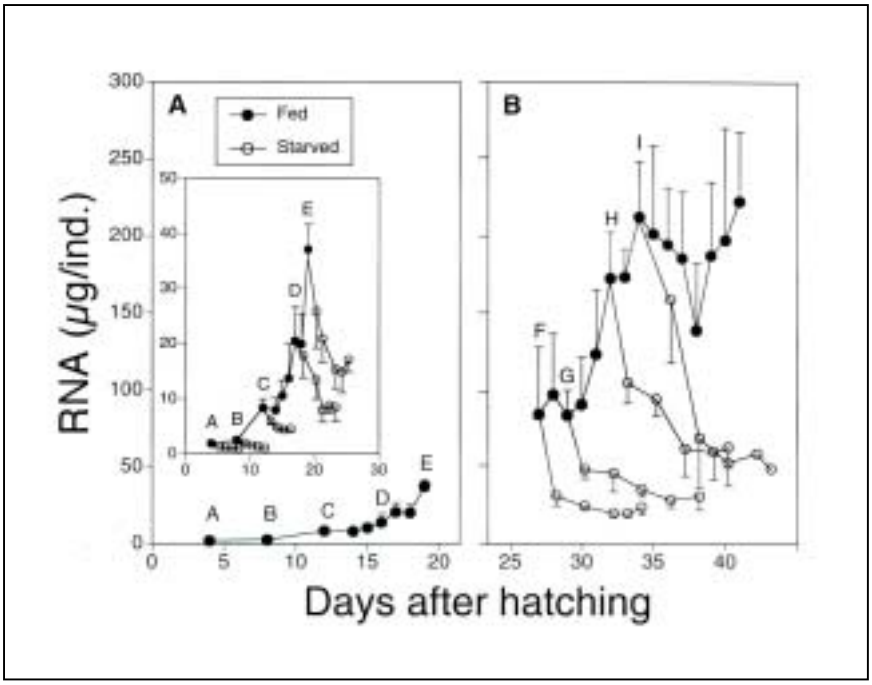


Figure 7. Developmental changes in RNA content of fed larvae and juveniles, and effects of starvation on the content in 1st (A) and 2nd (B) laboratory-reared Japanese flounder. Each point is the mean value of six fish. Vertical bars denote standard deviation, and A to I represent the developmental stage.

RNA: DNA, Protein: DNA, and RNA: Protein

The RNA: DNA for the fed group in Exp.1 showed, during the pre-metamorphic phase (A-E stages), a prominent increase between day 8 and 14, followed by a fluctuation until day 17 of D stage, and then reached a relatively steady level by day 22 of F stage (Fig. 8A). The RNA: DNA exhibited a marked fluctuation depending on developmental stage or day during the metamorphic (F to H) and post-metamorphic phases (I). The RNA: DNA obtained in Exp.2 showed a more stable pattern (Fig. 8B). It increased slowly between early and mid metamorphic stages (F and G), and reached the highest value of 5.36 ± 0.62 at the H stage. Thereafter, the value dropped to 2.49 ± 0.14 (I_5 stage: 5 days post-settlement), and then increased to 3.08 ± 0.25 at I_8. During larval starvation the ratio dropped consistently at every stage. For example, at the D stage it fell from 3.50 to 1.16 for five days, corresponding to 50% larval mortality (Fig. 8A).

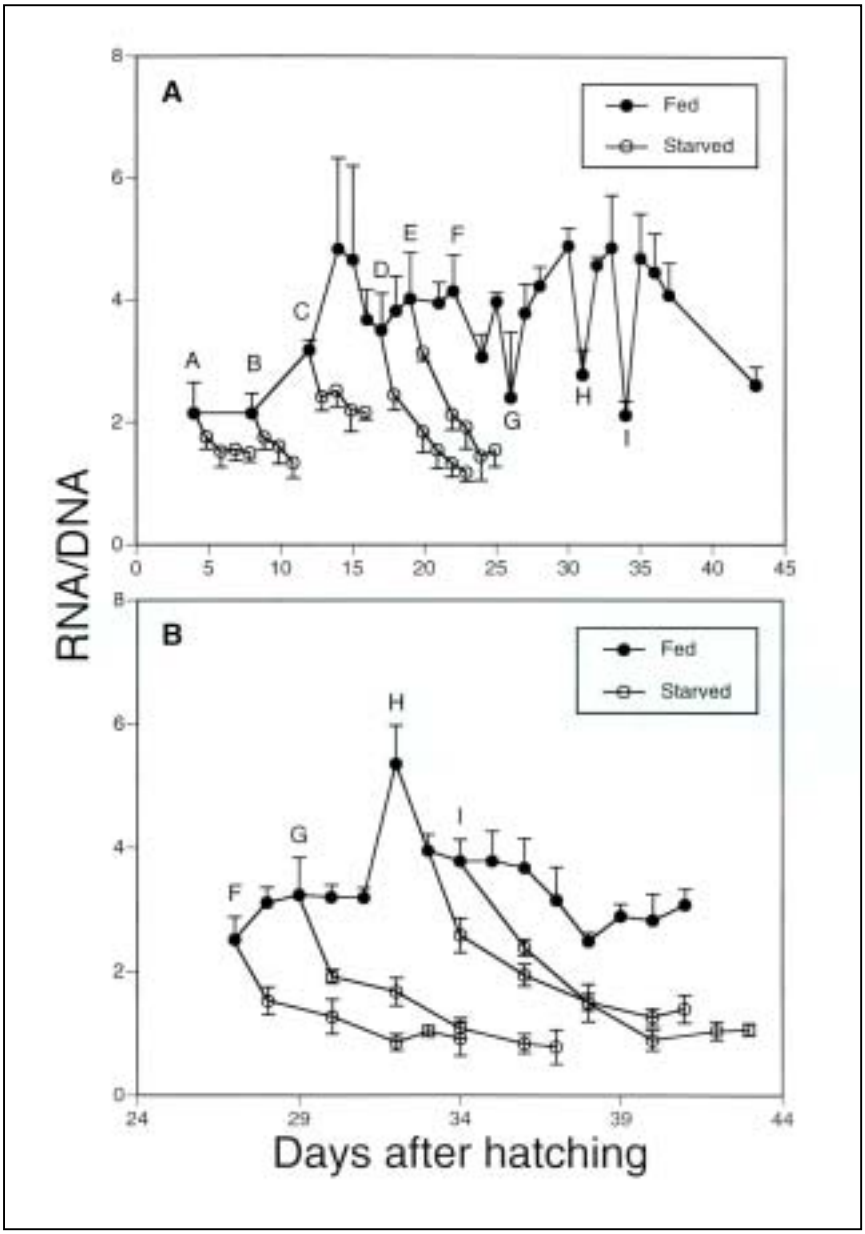


Figure 8. Developmental changes in RNA/DNA ratios of laboratory-reared Japanese flounder larvae and juveniles and changes in the ratios induced by starvation. A: 1st experiment, B: 2nd experiment. Vertical bars denote standard deviation, and A to I represent the developmental stage.

In Exp.3, RNA: DNA showed two peaks that occurred after onset of metamorphosis and at metamorphic climax stage H with the highest value of 5.07 ± 0.66 (Fig. 9). The RNA: DNA drastically dropped to 2.41 ± 0.85 at I_6 stage, and then tended to increase again. A gradual increase in DNA content versus a stable level of RNA content between late metamorphic and I_6 stages (Fig. 4A), caused an overall significant decrease in RNA: DNA. However, it increased again from I_6 stage with a relatively higher increase in RNA.

Protein: DNA also peaked at I stage resulting mainly from a higher rate of increase in protein content. The ratio then decreased markedly until the completion of metamorphosis, probably due to a slow increase in protein content, and a higher increase in DNA content (Fig. 10). Protein: RNA showed a drastic decrease at the early metamorphic stage and remained constant between mid and late metamorphic stages, followed by a slight decrease at post-metamorphic (Fig. 10).

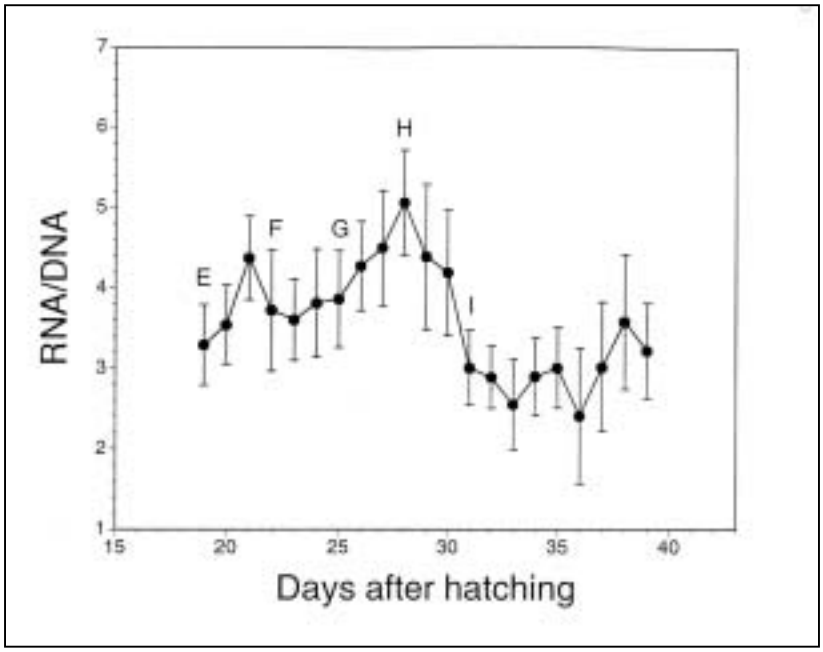


Figure 9. Developmental changes in RNA/DNA ratios of Japanese flounder larvae and juveniles during metamorphosis and early postmetamorphic phase (supplemental 3rd experiment). Values are given as the mean ± SD of five to eight fish. E to I indicate developmental stage.

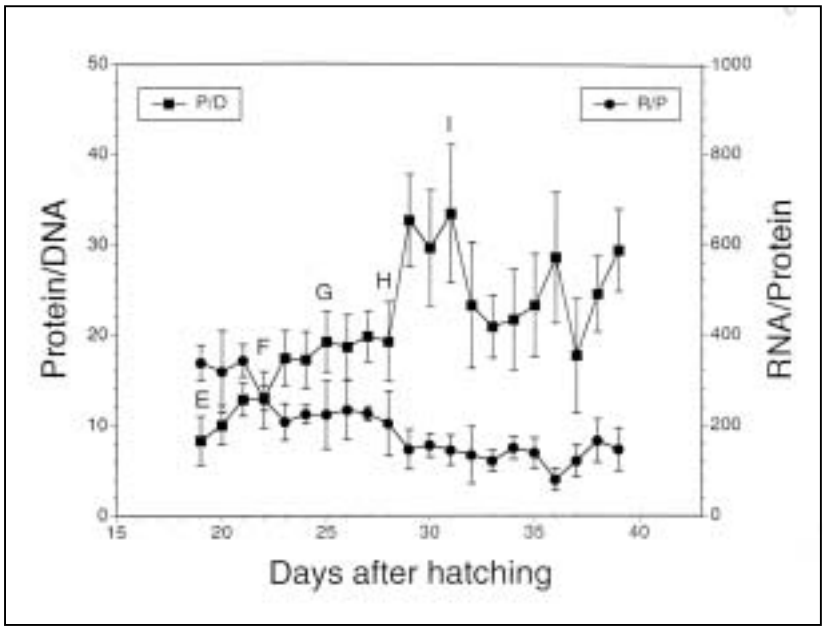


Figure 10. Developmental changes in protein/DNA and RNA/protein ratios of Japanese flounder larvae and juveniles during metamorphosis and early postmetamorphic phase (supplemental 3rd experiment). Values are given as the mean ± SD of five to eight fish. E to I indicate developmental stage.

Table 1 shows the nutritional criteria for evaluating the conditions of wild fish established by RNA/DNA ratios in laboratory rearing experiments. Each were classified either "healthy," if the RNA: DNA was as high as the well-fed hatchery-reared fish, "starving," if the RNA/DNA ranged between that of the well-fed and starved fish during the point-of-no-return (50% mortality), or "dying," if the ratio was below that of the point-of-no-return fish.

The RNA: DNA for wild larvae and juveniles are shown in Fig. 11. Forty-eight pre-metamorphic larvae (B-D stages) were caught in coastal waters from 20 to 113m in depth and 39 late metamorphic larvae and juveniles were caught in the near-shore water ranging from 3.5 to 10m (Table 2). Although individual values varied, the trend indicates a developmental stage-dependent increase in RNA: DNA until H stage, followed by a decrease at I stage. RNA: DNA differed significantly between developmental stages (Fig. 11B, $P<0.0001$). Tukey means comparisons indicated that stage H, with the highest RNA: DNA differed significantly from all other stages, and that stage G also differed significantly from stage I. The ratios of pre-metamorphic phase larvae ranged from 2.7 to 9.4 with an increase in median value at each stage during the two cruises in 1994 (Fig. 11A). Developmental stages differed significantly in their ($P=0.002$). Tukey means comparisons indicated that stage B, with the lowest RNA: DNA, differed significantly from stages E and G, which had the highest ratios, and that stage C also differed significantly from stage E. The general pattern of changes in RNA: DNA for the metamorphic

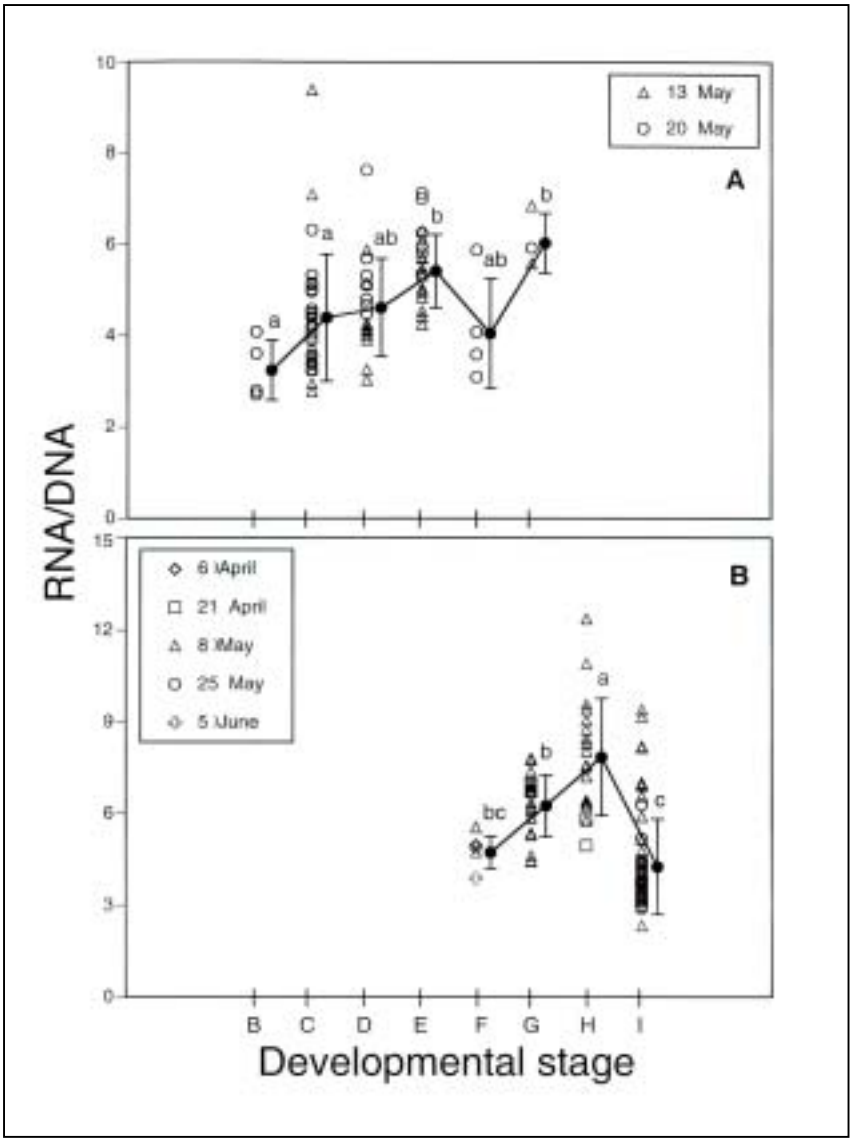


Figure 11. RNA/DNA ratios of wild Japanese flounder larvae and recently settled juveniles collected in the western Wakasa Bay in 1994 (A, N=75) and 1995 (B, N=112). Each closed circle with vertical bar shows the mean value and standard deviation of wild fish at each developmental stage. Vertical bars with same letter are not significantly different ($P<0.05$).

wild larvae was similar to that of the hatchery-reared fish (Fig. 11B). Of the 75 larvae (between C and G stages) collected in 1994, four C and D stage larvae were identified as "starving". Fish collected in 1995 consisted of advanced-stage (G and H stages) larvae and juveniles, but a relatively higher percentage of them considered "starving" (Table 2). The "starving" percentage (54%) at I stage was particularly high. The temperature at each sampling site ranged from 15-16 C in April 1995 to 17-18 C in May and June of 1995 (Table 2).

Discussion

The RNA: DNA of larval Japanese flounder show a drastic increase between 8 (B stage) and 13 DAH following a relatively stagnant state from 4 to 8 DAH (Fig. 8A). Compared with other species, these flounder are characterized by almost no increase for several days after the commencement of feeding, suggesting that a prolonged period of starvation-induced mortality exists. After successfully passing through the critical period at which a shift of energy source occurs, RNA: DNA prominently increases toward day 13, suggesting an occurrence of continuous cell divisions combined with increasing cell size or hypertrophy. Thereafter, the ratio markedly drops until day 17 due to a higher rate of increase of DNA content. This may be caused by greater cell proliferation than protein synthesis. These developmental patterns in RNA: DNA during the pre-metamorphic phase from A to E stages are in agreement with the results of Clemmesen (1987) for herring and turbot larvae, and with the work of Richard *et al.* (1991) on common sole.

Late larval and early juvenile Japanese flounder showed largely fluctuating RNA: DNA during metamorphosis and post-settlement, mainly as a result of fluctuating RNA content versus a relatively gradual increase in DNA content during Exp.2 and 3. In Exp.3, DNA content increased rapidly until 24 DAH, increased slowly at the mid- and late metamorphic stages (G and H) and then rapidly again. This ontogenetic pattern in DNA content appears to be amplified by that of RNA (Fig. 9). It indicates that the early ontogeny of Japanese flounder is composed of cyclic phases of hyperplasia and hypertrophy. Fukuda *et al.*, (1986) reported this kind of cyclic phase in the larval growth of cresthead flounder *Limanda schrenki*. Takii *et al.* (1994) also described a similar result in striped jack *Caranx delicatissimus*. It could therefore be postulated that both RNA and DNA contents are generally more closely linked to developmental stage than to age in larval and juvenile Japanese flounder. Richard *et al.* (1991) made such observations in *Solea solea.* Ehrlich (1974) and Love (1980) also supported the theory that chemical changes are more closely dependent upon larval size than upon age.

A drastic increase in RNA content between the early and late metamorphic stage results in marked increase in protein content and a peak of RNA: DNA. After the peak at the late metamorphic stage (H), the ratio decreased promptly until I_6 stage, primarily due to a drastic increase in DNA versus a decrease in RNA. Similar change was observed by the end of metamorphosis in the plaice larvae (Christensen and Korsgaard, 1999). An increase in RNA: DNA and protein: DNA was repeatedly reflected in the increased dry weight. Greater increasing rates in RNA content during days 19–28 and in DNA content during days 30–39 correspond to hypertrophy and hyperplasia phases of growth in the Japanese flounder. During increases in RNA: DNA it could be speculated that body growth of larvae chiefly occurs by cell enlargement (hypertrophy) resulting from active protein synthesis (Fukuda *et al.*, 1986).

Similar changing patterns in both protein and RNA contents were confirmed during metamorphosis. Protein content leveled off as RNA content decreased between late metamorphic

and post-metamorphic phases. The ontogenetic pattern in protein content during metamorphosis and post-settlement corresponds to those of RNA, indicating that RNA content reflects protein synthesis. A steep increase in both RNA and protein after the post-metamorphic phase was also observed in the DW gain. This fluctuation has led to a marked change in protein: DNA and RNA: DNA around 30 DAH. A decrease in protein: DNA during settlement coincides well with the result of cresthead flounder (Fukuda *et al.*, 1986). Moreover, a marked decrease in RNA: protein between the late and post-metamorphic phases suggests that protein synthesis drop during the non-feeding, settlement period (Tanaka *et al.*, 1996). Thus, energy reserves would be required to complete metamorphosis. These findings indicate that active cell enlargement (hypertrophy) may occur between early and late metamorphic stages, and is followed by higher cell proliferation (hyperplasia) and reduced protein synthesis during the post-metamorphic phase. They also provide evidence for a characteristic cyclic phase of hyperplasia and hypertrophy during metamorphosis. In addition, highly accumulated glycogen in the hepatic tissues was observed during the early metamorphic stage, contrasting to vacuolated hepatocytes found during the post-metamorphic phase (Gwak *et al.*, 1995). It is therefore possible that Japanese flounder larvae save energy by actively synthesizing protein until the metamorphic climax (stage H.) in order to cope with a short-term non-trophic period following settlement.

On the contrary, decreases in RNA: DNA from late metamorphic to post-metamorphic phases could be explained by a higher cell proliferation (hyperplasia) and/or a period of no change in RNA content, resulting from the non-trophic phase. Metamorphic transformation mainly involves tissue degeneration by eventual loss of larval structure, proliferation from larval tissues and organs into those of the adult and the formation of new adult tissues and organs from primordium (Youson, 1988). RNA and DNA dynamics during metamorphosis would correspond well to these degeneration-proliferation developmental events.

RNA content per individual fish dropped drastically throughout the entire starvation phase (Fig. 7), while the DNA content increased or decreased depending upon developmental stage (Fig. 6). Therefore, RNA: DNA dropped due primarily to a much higher reduction rate of RNA content. In fact, starved I stage fish had only 76% of the RNA content of fed fish at I stage. This resulting decrease in the starved larvae and juveniles occurred from the onset of starvation to the day of point-of-no-return. Under deprivation of food supply, a significant amount of RNA could be metabolized at onset, while DNA remained unaffected or slightly increased.

Clemmesen (1987) and Raae *et al.* (1988) made similar observations that DNA content increased with starvation for herring, turbot and cod larvae. Raae *et al.* (1988) also suggested that higher DNA content of starved larvae might be the result of residual cellular energy being used for rapid, unscheduled DNA synthesis, as cellular control mechanisms degenerate due to the lack of sufficient nutrition. Application of RNA: DNA for the assessment of larval nutritional condition has been based on a constant level of DNA under insufficient food supply, as well as significant reduction of RNA. Although there is a large difference between fed and starved fish at 37 DAH with regard to these ratios and total length (Gwak *et al.*, 1999), morphological appearance was similar. Consequently, it is possible that during metamorphosis starved fish mainly utilize their limited energy to maintain metabolism and complete metamorphosis. The increase of DNA content during starvation conditions should be examined in more detail from various aspects, such as species-specific, development-specific, biotic and abiotic factors.

In 1994, all wild larvae were considered healthy except four larvae collected on 13 May, which were determined as starving. In 1995 over 65% starving larvae and juveniles were recognized, all in the H and I stages. Additionally, most were collected in shallower water,

during the late settling season of 25 May and 5 June (Table 2). Since Japanese flounder larvae and juveniles (E-I stages) reared under different temperatures (14 and 22 C) with enough food showed no significant difference in RNA: DNA (Gwak, 1999), when utilizing the established criteria we may negate the temperature effects caused by differences between sampling site and rearing experiment. Tanaka *et al.* (1996) noted a high incidence of empty stomachs in newly settled wild juveniles. This indicate that late larvae and early juveniles caught in shallower water during the late settling season could be more vulnerable to starvation than earlier ones, presumably owing to lower food availability and higher metabolic rate under higher water temperature. Recently settled Japanese flounder prey newly emerged mysid larvae, where abundance in the nursery appears to be variable (Tanaka *et al.*, 1989b). Mysid abundance seems to markedly decrease with the settling season and/or increasing temperature in the Wakasa Bay area (Yamaguchi *et al.*, unpublished data). These field results correspond well to the results obtained in the laboratory where abrupt decreases in RNA: DNA occurred at settlement. They also support the presence of a second critical phase associated with settlement, which can be speculated from ontogenetic changes in the RNA: DNA of hatchery-reared fish.

Changes in feeding and food habits before and after metamorphosis seem to vary with species or type of metamorphosis and/or settlement in flatfishes. Hotta *et al.* (unpublished data) recently demonstrated that a pleuronectid spotted halibut *Verasper variegatus* shows a different type of metamorphosis with continuous feeding during gradual settlement without any sign of feeding cessation. There is no clear reduction of RNA: DNA at settlement. The reduction in Japanese flounder may be more of an ontogenetic nature than environmental effect.

The present study shows that RNA, DNA, and protein content are reliable criterion to investigate the ontogenetic changes during early life history of Japanese flounder. Higher sensitivity of RNA to starvation suggests that RNA: DNA is a good indicator for nutritional condition of larval and juvenile Japanese flounder.

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